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Deletions in the Hepatitis B Virus Core Gene May Influence the Clinical Outcome in Hepatitis B e Antigen–Positive Asymptomatic Healthy Carriers

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To address the significance of mutations within the hepatitis B virus (HBV) core gene in chronic HBV infection, we followed prospectively HBe-antigen–positive asymptomatic healthy carriers, documented the onset of their disease based on serum alanine transaminase (ALT) concentrations, and analyzed sequentially serum samples from a quiescent phase through to an active phase of the chronic infection. In three female carriers, the first flare-up was documented during the follow-up period. Serial analysis by polymerase chain reaction, cloning, and sequencing of the HBV precore/core open reading frame genome demonstrated that clones with core gene deletions emerged during the quiescent phase and persisted subsequently during the active phase in two patients, who failed to seroconvert to anti-HBe and had persistently increased ALT levels despite interferon (IFN) therapy. The deletions were various, overlapping, and located in the mid-core region ranging from amino acid (aa) position 64 to 128. The remaining patient, who seroconverted with IFN therapy, did not have a core-gene-deletion HBV variant during follow-up, but had aa substitutions clustered in some restricted core regions. Two control asymptomatic carriers, who had no change in biochemical or virologic markers over a 15- to 19-year period, had no core-gene-deletion variants and few aa changes. These findings indicate that the mid-portion of the core gene is subject to deletion even during the quiescent phase. Thus, the immunologic interaction between the host and virus may occur insidiously, and the emergence of a core-gene-deletion HBV variant during the quiescent phase may be involved in the onset of hepatitis and the subsequent outcome of chronic infection. ***J. Med. Virol. 56:287–293, 1998.***

KEY WORDS: HBV core gene deletion; HBeAg-positive asymptomatic carriers; serial analysis by cloning and sequencing; quiescent phase of chronic HBV infection

Introduction

In the natural course of chronic hepatitis B virus (HBV) infection, the initial phase represents a quiescent immune tolerance characterized by high levels of viremia but with normal concentrations of serum aminotransferase and minimal or no inflammation by liver histology. Subsequently, there is a transition to an active phase of chronic infection and eventually seroconversion from an HBe-antigen (HBeAg) –positive state to anti-HBe–positivity accompanied by an exacerbation of hepatic injury [Hoofnagle et al., 1981; Chu et al., 1985]. During the active phase, the cellular immune response in the HBV-induced immunopathology is primarily responsible for the HBV-infected hepatocellular damage, resulting in viral elimination [Naoumov and Eddleston, 1994]. The immunopathogenic mechanisms involved in the evolution of the chronic disease through these different phases is not fully understood.

In chronic HBV infection, hepatitis B core antigen (HBcAg) encoded by the HBV core gene has been shown to be an important target for the T lymphocyte-mediated lysis of HBV-infected hepatocytes [Mondelli et al., 1982]. The lymphocytes isolated from chronic HBV-infected patients exert a selective immune pressure against hepatocytes that express HBcAg [Naumov et al., 1984]. The significance of HBcAg in the activation of the T lymphocyte response was supported by the observation that HBcAg-specific $CD4^+$ helper and $CD8^+$ cytotoxic T lymphocytes are present in the peripheral blood [Ferrari et al., 1987a] and within the liver [Ferrari et al., 1987b] of patients with chronic

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active hepatitis B. Furthermore, the amino acid (aa) sequence of HBcAg overlaps in the greater part with that of HBeAg coded for precore/core open reading frame (ORF), a critical marker for seroconversion to anti-HBe in the course of HBV infection [Uy et al., 1986; Standring et al., 1988]. These studies led us to examine whether the HBV core gene coding for HBcAg holds an important clue in determining the natural course and outcome of the chronic infection.

Several studies have demonstrated that clustered aa mutations or deletions in a restricted region of the HBV core gene appeared in chronic HBV carriers [Okamoto et al., 1987; Wakita et al., 1991; Ehata et al., 1992; Ackrill et al., 1993; Akarca and Lok, 1995; Günther et al., 1996; Marinos et al., 1996]. These findings suggest that the particular locations of aa alterations within the core gene are likely to be preferential sites recognized by the host immune system. However, it is still not known how the genome sequence in such regions changes longitudinally from a quiescent to an active phase. It is not clear when the aa substitutions or deletions occur in the HBV core gene, or how the occurrence of these variants may affect chronic infection. The changes in the core sequence, which may occur during the quiescent phase, may reflect the pathogenesis of the disease or influence the natural course and outcome of the chronic infection.

To address these issues, we analyzed prospectively the serial nucleotide changes within the HBV core gene in asymptomatic healthy carriers as they developed their first onset of hepatitis.

MATERIALS AND METHODS

Patients

To identify the first onset of hepatitis based on serum alanine transaminase (ALT) in asymptomatic healthy carriers, we followed prospectively HBV-infected carriers whose infection route was considered to be mother-to-infant transmission. In three female patients, the first ALT flare-up was documented in detail during the follow-up period (Fig. 1). They all received interferon (IFN)-α therapy for chronic HBV infection after the onset of the disease. Human lymphoblastoid IFN-α (Sumitomo Pharmaceuticals Co. Ltd., Tokyo, Japan) was given intramuscularly (6 MU, twice weekly for 4 months). As controls, two other female patients who had normal ALT levels during the entire follow-up period (15 and 19 years) were studied. Serum samples were taken serially at four to six different time points from the former and two different time points from the latter. At the initiation of the follow-up, they were seropositive for both HBsAg and HBeAg and were followed prospectively with clinical and laboratory data every 1 to 3 months. All samples were negative for anti-HCV antibody measured by a second-generation EIA kit (Ortho Diagnostic Systems, Raritan, NJ). Serum samples were stored at −80°C until examined.

Serologic Markers of HBV

HBsAg, anti-HBs, HBeAg, and anti-HBe were assayed by commercially available RIA systems (Abbott Laboratories, Chicago, IL). HBV-associated DNA polymerase (DNA-P) activity was measured by determining [^{3}H]thymidine incorporation. Subtypes of HBsAg were determined by solid-phase EIA (Institute of Immunology, Tokyo, Japan). All five patients had HBV subtype *adr*.

DNA Extraction and Polymerase Chain Reaction (PCR) Assay

HBV-DNA was extracted from the sera by a conventional sodium hydroxide procedure. Serum proteins were denatured by the addition of 20 μL of 0.2 N NaOH to 20 μL of serum. After 1-hour incubation at 37°C, the samples were neutralized with HCl and then centrifuged for 5 minutes. A 1-μL aliquot of supernatant was amplified subsequently by PCR in a 25-μL mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 10 pmole of each PCR primer, 200 μM each dNTP, 0.01% gelatin, and 1 unit of Taq polymerase (Takara Shuzo, Kyoto, Japan) for 35 cycles. Each cycle consisted of denaturing at 94°C for 1 minute, annealing of the primers at 55°C for 1 minute, and extension at 72°C for 3 minutes. Five microliters of the PCR products were analyzed by electrophoresis using 2% agarose gels, stained with ethidium bromide, and visualized under UV light. The sense and anti-sense synthesized oligonucleotide primers were CE1 (5′-TTGTACTAGGAGGCTGTAGGC-3′, nt 1768–1788, numbered according to Okamoto et al., 1986) and CoR3 (5′-TCCCACCTTATGAGTCCAAG-3′, nt 2458–2477), respectively. The PCR amplification product of HBV DNA by the primer set was 710 bp in length and covered the entire precore/core open reading frame. To prevent false-positive results, the contamination-avoidance guidelines were applied strictly and negative controls were included in each assay.

Cloning and Sequencing

The PCR products were inserted by blunt-end ligation into EcoR V–digested phagemid pBluescript II™ (Stratagene, La Jolla, CA) with the addition of a single thymidine at the 3′-end of each fragment [Marchuk et al., 1990]. After the ligation reaction, the recombinant phagemids were introduced into competent *Escherichia coli* JM109 (Wako Pure Chemical Industries, Osaka, Japan) and cloned. Five to 12 clones were randomly and independently selected from the clones propagated from the sera. Fluorescence-based sequencing was undertaken in both directions using a 373A DNA Sequencing System™ and Taq Sequencing Kit™ (Applied Biosystems, Foster City, CA). A computer-assisted homology search was conducted by DNASIS™ Ver. 7.00 (Hitachi Software Engineering, Tokyo, Japan). To assess the frequency of aa substitutions in each clone, the number of aa residues different from the reported sequence of wild-type HBV in subtype *adr* [Fujiyama et al., 1983; Ono et al., 1983; Kobayashi and Koike, 1984] was counted for each clone.

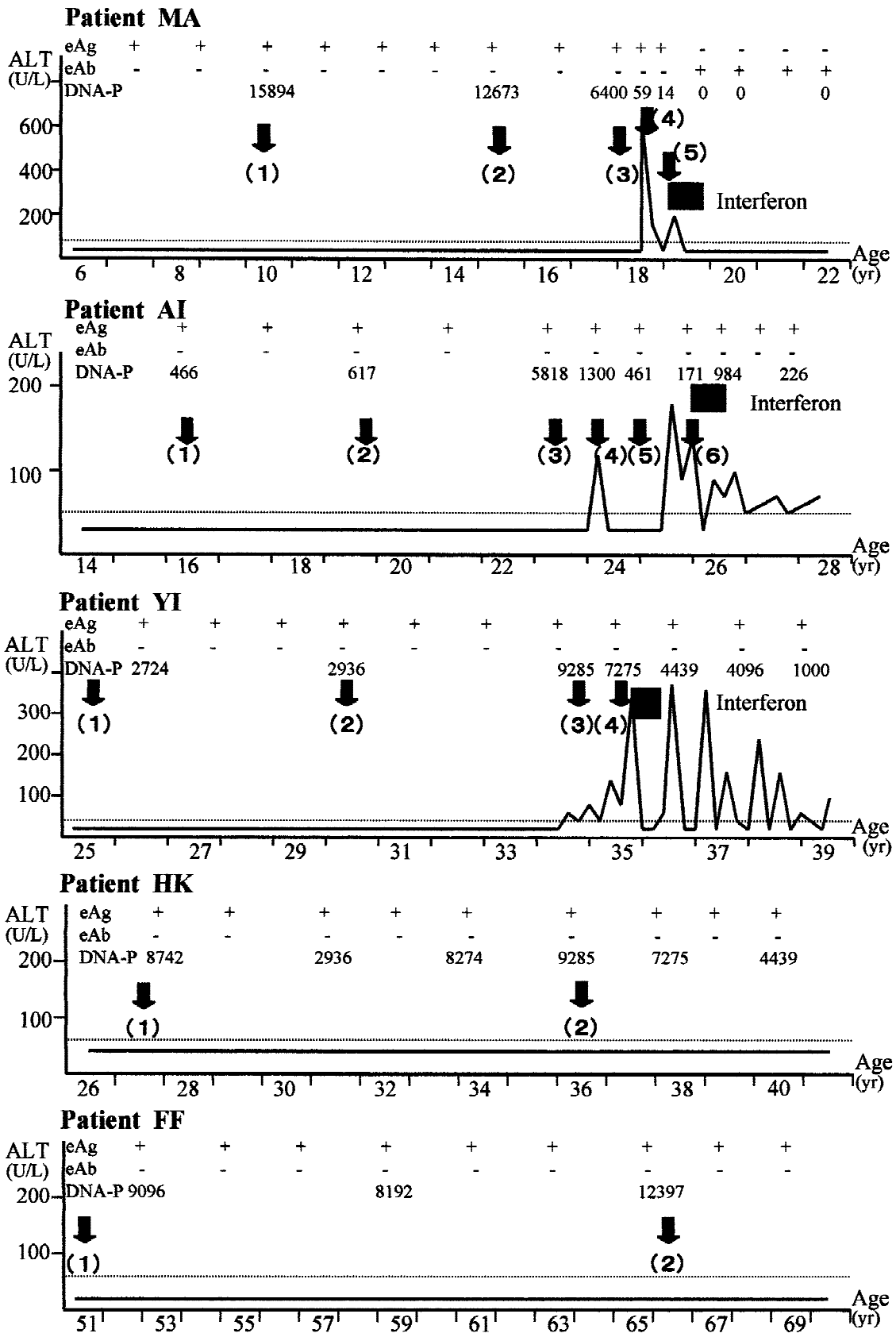

Fig. 1. The clinical course and virologic transition of five HBeAg-positive asymptomatic carriers. Patients MA, YI, and AI showed the first onset of hepatitis during their disease courses. Patient MA succeeded in HBeAg-to-HBeAb seroconversion with interferon therapy, whereas patients YI and AI failed to seroconvert despite treatment. Control carriers HK and FF had no change in biochemical and virologic markers during long-term follow-up. The individual serum samples for cloning and sequence analysis are indicated with arrows, and the number associated with the arrows denotes the sequential time point for each patient.

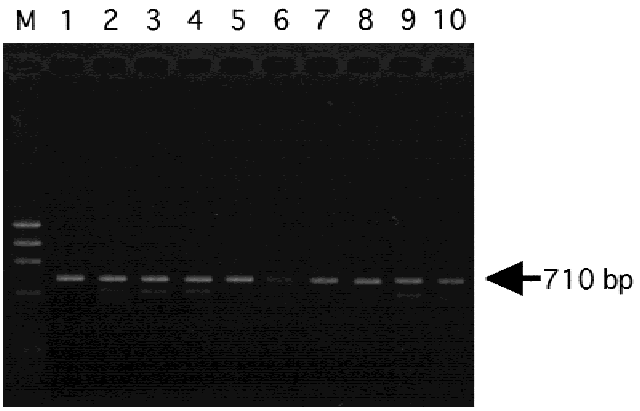

Fig. 2. Ethidium-bromide–stained agarose gel of PCR amplification products of the precore/core HBV DNA genome in patients AI and YI. **Lanes** are as follows: **1–6,** patient AI; **7–10,** patient YI; **M,** molecular size markers (ϕX174RF DNA HaeIII digest). Lanes 1 and 7 show a single band of the expected size (710 bp). Lanes 2–6 and 8–10 had a few shorter bands in addition to the expected bands.

RESULTS

Clinical and Virologic Characteristics of Patients

The long-term clinical course and virologic features of the five patients surveyed in this study are illustrated in Figure 1. Three of the five patients, patients MA, AI, and YI, received IFN therapy after the onset of hepatitis. They were continued histologically to have chronic active hepatitis (CAH) by liver biopsy just before treatment. All serum samples analyzed for cloning and sequencing were obtained before treatment. Patient MA seroconverted successfully from HBeAg positivity to anti-HBe positivity with IFN therapy. At the same time, the level of DNA polymerase activity declined dramatically and continued subsequently to be negative, resulting in the subsidence of hepatitis. In patients AI and YI, HBeAg remained seropositive, and DNA polymerase activity levels fluctuated despite IFN therapy. They continued to have elevated serum ALT levels after the completion of treatment. The control asymptomatic carriers, HK and FF had normal ALT levels constantly over a 15- or 19-year follow-up period, respectively. In these controls, virologic markers were also unchanged.

Polymerase Chain Reaction Amplification of the Precore and Core Open Reading Frame

In all sera derived from patient MA, each PCR product was sharply visible as a single DNA band of the expected size on the ethidium-bromide–stained agarose gel. However, gel electrophoresis of amplification products showed the presence of additional faint shorter DNA bands in the serum samples obtained at time points 2, 3, 4, and 6 in patient AI, and at time points 2, 3, and 4 in patient YI, although the expected-sized DNA band was detected vividly in all the PCR products except one (Fig. 2). At point 6 in patient AI, the expected-sized band was more faint than those at other points in the same patient or the other two patients. At point 5 in patient AI, only a single expected-sized band was detected. In all the sera from both controls, HK and FF, only a single DNA band was sharply visible at the expected size, and additional shorter bands were not observed at all.

Changes of Deduced Amino Acid Sequences of the Precore and Core Genes

The number of deduced aa changes per clone in the precore and core sequences is depicted in Table I. Most aa substitutions were not common among clones analyzed in this study. They were scattered over the precore and core sequences (data not shown). To exclude sequence changes introduced artificially during PCR, only differences that were seen in at least two clones are presented (Table I). In patient MA without a deletion variant, we found the precore stop codon as well as mutations common to clones around the onset of disease (Fig. 1; Table I). The deduced aa changes were concentrated in $CD4^+$ helper T-lymphocyte epitopes at aa 1–20 and 117–131 [Ferrari et al., 1991], the mutation cluster region at 84–110 [Ehata et al., 1992], and the arginine-rich domain at 150–161 [Hatton et al., 1992; Nassal, 1992]. In contrast, patients AI and YI, in whom deletion variants emerged during follow-up, had no mutation clusters at these positions.

Variants With Deletion Within the Core Gene

Clonal analysis of amplified DNA obtained from patient AI's sera showed that two of six independent clones at time point 2 during the quiescent phase had in-frame deletions of 87 and 135 bp leading to losses of aa 78–106 and 84–128 of the core gene, respectively (Table I). Subsequently, three of five, two of five, and five of five clones at points 3, 4, and 6, respectively, had various in-frame deletions. Similarly, patient YI also had various in-frame deletions in the follow-up samples from the quiescent phase through the active phase; two of eight clones at point 2, two of seven at point 3, and seven of nine at point 4. Although all of the clones analyzed at point 6 in patient AI had deletions, gel electrophoresis demonstrated the existence of the expected-sized, although much fainter, band compared to those in other samples. Other clones derived from these patients had complete precore/core ORF sequence. Accordingly, the results of cloning and sequencing were in accordance with those of the PCR. Furthermore, the core-gene–deletion HBV strains had already emerged during the quiescent phase before the first onset of hepatitis. In addition, they had no precore stop mutation and no mutation cluster in a specific restricted region of the core gene. All clones isolated from patients MA, HK, and FF had no deletion within the precore/core ORF. Accordingly, they included the full precore/core genomes.

DISCUSSION

In this study, HBV variants with core gene deletions were detected sequentially through the quiescent phase to the active phase of the chronic infection. Clon-

TABLE I. Deletion and Substitution of Amino Acids in the HBV Precore/Core Open Reading Frame in Sequential Samples From HBeAg–Positive Asymptomatic Carriers*

			Precore region		Core gene		
Patient	Time points	Total No. of clones	No. of aa changes per clone[a]	No. of stop mutant[b]	No. of aa changes per clone[a]	Position and type of aa substitution[c]	AA deletion position[d]
MA	1	9	0–1 (0)	0	0–2 (0)	—	—
	2	6	0	0	0–2 (0)	—	—
	3	9	0–2 (1)	5	3–8 (4)	^{13}V→A (7), ^{97}I→L (5), ^{100}L→I (5), ^{130}P→T (5), ^{151}R→C (4), ^{153}G→C (5), ^{156}P→T (3)	—
	4	6	0–1 (0)	0	2–7 (3)	^{97}I→L (2), ^{100}L→I (2), ^{130}P→T (2), ^{151}R→C (3), ^{153}G→C (2)	—
	5	9	0–1 (0)	3	2–7 (4)	^{13}V→A (5), ^{97}I→L (5), ^{100}L→I (5), ^{130}P→T (4), ^{151}R→C (4), ^{153}G→C (3)	—
AI	1	5	0	0	1–6 (2)	—	—
	2	6	0–1 (0)	0	0–3 (2)	—	78–106 (1), 84–128 (1)
	3	5	0–1 (0)	0	0–3 (0)	—	78–103 (1), 78–105 (1), 76–106 (1)
	4	5	0–2 (0)	0	0–1 (1)	—	79–107 (2)
	5	5	0–1 (0)	0	0–1 (0)	—	—
	6	5	0	0	1–3 (1)	^{122}F→V (2)	79–107 (1), 78–109 (1), 75–114 (1),78–118 (1), 64–110 (1)
YI	1	8	0–1 (0)	0	0–3 (0)	—	—
	2	8	0–1 (0)	1	0–3 (1)	—	79–107 (2)
	3	7	0	0	0–3 (2)	^{29}D→G (2)	77–104 (1), 74–106 (1)
	4	9	0–1 (0)	0	0–4 (1)	^{21}S→A (2), ^{130}P→T (2)	80–107 (1), 87–108 (2), 79–107 (2), 84–119 (2)
HK	1	12	0–2 (0)	1	0–1 (0)	—	—
	2	9	0–1 (0)	0	0–2 (1)	—	—
FF	1	12	0–1 (0)	0	0–2 (1)	—	—
	2	10	0–1 (0)	0	0–4 (0)	—	—

[a]The number of amino acid (aa) changes is expressed as the range and median in parentheses.
[b]Precore stop mutation is substitution from G^{1896} to A^{1896}, converting from tryptophane^{28} to stop codon^{28}.
[c]The left superscript and the number in parentheses refer to the aa position from the amino terminus of the core protein and the number of clones, respectively.
[d]Parentheses indicate number of clones with each deletion.
*Abbreviations for each aa are shown in standard nomenclature: A, alanine; C, cysteine; D, aspartic acid; F, phenylalanine; G, glycine; I, isoleucine; L, leucine; P, proline; R, arginine; S, serine; T, threonine; V, valine.

ing and DNA sequencing analysis showed that the deletions were all in-frame, but various and overlapping even in the same patient or sample, resulting in the loss of 26–47 aa in length when compared to the wild-type consensus sequence. The various overlapping deletions were located between aa 64 and 128 around the mid-portion of the core gene, and thus did not overlap with the X and polymerase ORFs. In addition, the deletion position did not harbor some *cis*-acting elements required for genomic replication and viral DNA synthesis: ecapsidation signal ε [Junker-Niepmann et al., 1990], direct repeat 1, polyadenylation signal [Will et al., 1987], and arginine-rich carboxy-terminal domain [Hatton et al., 1992; Nassal, 1992]. A recent in vitro study, however, has shown that such deletion mutants alone are replication defective at the RNA packaging level [Yuan et al., 1998]. The core deletion genomes could be rescued by *trans*-complementation from core-gene–intact wild sequences, resulting in a replication level similar to that of the wild type [Horwich et al., 1990; Okamoto et al., 1993; Yuan et al., 1998]. The mutants rescued could be also secreted from the transfected cells into the medium and existed in the rescued and secreted viral particles, which were similar to the mature wild-type Dane particles. Thus, our patients had no change in HBe status or viral replication before or after the emergence of the deletion mutants, because they were shown to have coexistent complete genomes. Alternatively, absence of the J-ATG and C2-ATG start codons, which are located within the deletion position and initiate the translation of the HBV polymerase gene from the pregenomic RNA in the ribosomal scanning mechanism model [Fouillot et al., 1993], may offer some advantage over viral replication and synthesis in persistence of infection. The two initiation codons hamper the expression of viral polymerase.

Other investigators have shown that core gene deletions were located within aa 81–113 [Ackrill et al., 1993], 79–112 [Akarca and Lok, 1995], and 63–132 [Wakita et al., 1991]. The similar mid-portion of the core gene is likely to be subject to deletion. The common deletion position in this study was at aa 87–103, and those in the three groups were at aa 88–105, 85–107, and 84–118. Interestingly, these common deletions have remarkably similar boundaries. Furthermore, the positions coincided with a cluster region of aa substitutions between aa 84 and 101 [Ehata et al., 1992], where patient MA persistently had mutations

around the flare-up of hepatitis, although the discrepancy between deletion and aa substitution could not be accounted for.

The location of the deletion described in this study contains two B-lymphocyte epitopes at aa 74–89 [Salfeld et al., 1989; Sällberg et al., 1991] and 107–118 [Colucci et al., 1988] and a $CD4^+$ helper T-lymphocyte epitope at aa 117–131 [Ferrari et al., 1991]. Although intrahepatic HBcAg-specific T-lymphocyte lines derived from CAH patients consisted of $CD4^+$ and $CD8^+$ subpopulations in similar proportions, an HBcAg-specific proliferative response was limited to the $CD4^+$ T-lymphocytes [Ferrari et al., 1987b]. In patients with chronic HBV infection, the helper T-lymphocyte response to HBcAg was shown to be significantly detectable and seemed to be involved in enhancing the immune-mediated liver damage and in activating the HBV-specific humoral immune reaction [Marinos et al., 1995]. It is generally accepted that both cellular and humoral immune responses are required for successful viral elimination, and that $CD4^+$ helper T-lymphocytes may induce cytotoxic T-lymphocytes and immunoglobulin synthesis by B-lymphocytes [De-Kruyff et al., 1993; Stuhler and Walden, 1993]. Loss of the mid-core region harboring such epitopes may help to evade viral clearance by altering the host immune system recognition. Alternatively, in vitro studies suggest that expression of wild-type HBcAg can be suppressed by interference with the concomitant core deletion proteins [Horwich et al., 1990; Scaglioni et al., 1994], thereby allowing for persistent viral infection.

The observation that the core deletion genomes coexist with the complete core genes during the quiescent phase in HBeAg-positive carriers resolves the conflicting reports that core-gene–deletion variants were isolated from HBeAg-positive patients with CAH, but not with minimal liver lesions [Wakita et al., 1991; Ackrill et al., 1993], and that HBeAg-positive asymptomatic carriers had core gene deletions [Okamoto et al., 1987]. It is possible that such asymptomatic carriers with core gene deletions may develop hepatitis in the near future. In addition, they may fail to seroconvert to anti-HBe and may have a poor response to IFN therapy. This suggestion corresponds to a previous report stating that patients with core gene deletions are not more likely to clear HBeAg than those with intact core genes [Akarca and Lok, 1995]. In long-term immunosuppressed renal transplant patients, the core-gene–deletion mutants were shown to accumulate, and their persistence may be associated with progressive liver disease [Günther et al., 1996]. In HBeAg-positive asymptomatic carriers with or without a weak immune response to HBV, the emergence of core gene deletions may be related to an unfavorable outcome in the course of chronic infection.

However, there were conflicting observations that HBeAg-positive patients with CAH carrying core-gene–deletion variants were more likely to seroconvert to anti-HBe than those carrying only intact core sequence [Wakita et al., 1991; Marinos et al., 1996]. Furthermore, the deletion variants did not confer resistance to IFN therapy, although they had a significantly lower level of viremia than wild-type genomes and likely developed during the active phase of the disease [Marinos et al., 1996]. Different biologic properties of various deletion mutants may manifest their clinical effects differently and may induce various magnitudes of immune responses, because the core-gene–deletion mutants in our study emerged during the quiescent phase, not the active phase, and were associated with high levels of viremia. The occurrence of a core gene deletion during the quiescent phase suggests the loss of immune tolerance or the insidious beginning of the host immune response to HBcAg, although the onset of hepatitis had not yet manifested biochemically. Conversely, if the host immune surveillance does not yet recognize the virus, the core gene deletion could occur spontaneously, and might provide better host immune recognition of the viral antigen. This study indicates that core gene deletion could not have resulted from vigorous responses of the host immune system or from IFN therapy.

Patients infected with HBV carrying the precore stop mutation were not found to have core gene deletions [Ackrill et al., 1993]. In this study, patient MA developed the precore stop mutation and aa substitutions concentrating in some specific restricted regions but did not have a core-gene–deletion variant during the follow-up. Conversely, the core-gene–deletion strains had no precore stop mutations and were less likely to have mutations. Although the reason is unclear, the dominant presence and persistence of the deletion mutant may make it difficult to seroconvert to anti-HBe in patients such as AI and YI, whose core-gene–deletion HBV had no precore stop mutation.

In conclusion, this serial analysis by cloning and sequencing the HBV genome demonstrated that HBeAg-positive asymptomatic healthy carriers developed deletions within the core gene even during the quiescent phase of the chronic infection and that the deletion mutants persisted subsequently during the active phase. The presence of core-gene–deletion variants may influence an outcome in the course of chronic HBV infection. To clarify the significance of core-gene–deletion mutants in chronic HBV infection, long-term sequential analysis from a quiescent phase to an active phase is required in a large number of HBeAg-positive asymptomatic healthy carriers. In addition, in vitro studies of the cellular immune response to deleted forms of core protein could elucidate the biologic properties of the core-gene–deletion HBV.

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